High carrier prevalence of combinatorial CYP2C9 and VKORC1 genotypes affecting warfarin dosing

**Background:** Polymorphisms in the cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase complex subunit 1 (VKORC1) genes significantly alter the effective warfarin dose. The CYP2C9*2 (430C>T), CYP2C9*3 (1075A>C) and VKORC1 -1639 G>A polymorphisms affect warfarin dose through altered metabolism (CYP2C9) and sensitivity (VKORC1). **Objective:** We determined the frequencies of SNPs in the CYP2C9 and VKORC1 genes in a clinical outpatient population and the carrier prevalences for a variety of genotype combinations to gauge the impact of these polymorphisms on warfarin dosage using published algorithms. **Method:** A total of 127 patients from an outpatient clinic at Hartford Hospital (Hartford, CT, USA) were genotyped for five SNPs in the CYP2C9 gene and seven SNPs in the VKORC1 gene using Luminex® technology. **Results:** The polymorphism frequencies were 10.2, 7.9 and 37.4% for the functionally deficient CYP2C9*2, CYP2C9*3 and VKORC1 -1639 G>A polymorphisms, respectively. Combining prevalence of combinatorial genotypes, 18% were carriers of both CYP2C9 and VKORC1 polymorphisms, 13% were CYP2C9 polymorphism carriers only, 42.5% were VKORC1 carriers only, and the remaining 27% were noncarriers for either gene. Based on published warfarin dosing algorithms, carriers of 1, 2, 3 and 4 functionally deficient polymorphisms predict reductions of 1.0 to 1.6, 2.0 to 2.9, 2.9 to 3.7, and 3.6 to 4.4 mg/day, respectively, in warfarin dose. **Conclusion:** Overall, 73% of the population carried at least one polymorphism predicting deficient warfarin metabolism or responsiveness and 18% were carriers for polymorphisms in both genes studied. Combinatorial genotyping of CYP2C9 and VKORC1 can allow for individualized dosing of warfarin amongst patients with gene polymorphisms potentially reducing the risk of accentuated responses and bleeding.

Warfarin is the most commonly prescribed oral anticoagulant in the USA, with over 19.5 million prescriptions written in 2006 [1]. Dosing remains a challenging task, as anticoagulation therapy using warfarin is influenced by many factors, both physiological and genetic. For instance a patient’s International Normalized Ratio (INR) varies with Vitamin K intake [2], age [3] and body size and gender [4].

Common polymorphisms in two genes, cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase complex subunit 1 (VKORC1), affect warfarin dose through different mechanisms. Polymorphisms of CYP2C9 include *2 and *3 which are associated with a decrease in enzyme activity to approximately 70 and 5% of the normal level, respectively [5–9]. The result is excess warfarin accumulation, elevated INR (>4.0) and possible hemorrhagic complications [10]. The variants have a significant impact on the body's ability to metabolize warfarin in Swedish, Japanese, Italian, North American, British Caucasian and Hong Kong Chinese populations [6,8,11–18]. The CYP2C9 status by itself accounts for approximately 15–20% of the variance in warfarin dose [13,18,19].

Warfarin exerts an anticoagulant effect through its inhibition of the vitamin K epoxide reductase multienzyme complex (VKOR), part of which is subunit 1, encoded by VKOR1 [20,21]. VKOR is responsible for converting vitamin K epoxide to vitamin K hydroquinone, which is an essential cofactor required for the carboxylation of clotting factors II, VII, IX and X [22]. A common polymorphism of the VKORC1 promoter sequence (-1639 G>A) results in decreased vitamin K epoxide reductase enzyme activity as shown by in vitro promoter luciferase activity assays [23]. Patients who are carriers of this polymorphism require a lower warfarin maintenance dosage. In addition, six rare coding sequence mutations result in syndromes of warfarin resistance [20,24]. In patients requiring anticoagulant therapy, the -1639 genotype can independently determine 20–25% of warfarin dose variance [18,24].

Together, the CYP2C9 and VKORC1 combinatorial genotypes may explain up to 45% of warfarin response variability [12,13,19,24].

In August 2007, the US FDA revised the warfarin label to include a pharmacogenomic subsection in which the metabolic impact of the CYP2C9 and VKORC1 gene polymorphisms is outlined [25]. The CYP2C9 and VKORC1 polymorphisms are additionally referenced in the ‘Precautions and Dosage’ and ‘Administration’ sections of the label, noting that the presence of polymorphisms in those two genes may lead to the need for more frequent INR monitoring and reduced warfarin doses [25].

Given the narrow therapeutic range of the INR among patients receiving warfarin therapy, dose estimate empiricism is risk prone. The consequences of over- or under-anticoagulation can be serious, ranging from life-threatening bleeding to cerebrovascular emboli, and require intensive treatment. Major and fatal bleeding events occur at rates of 7.2 and 1.3 per 100 patient years, respectively, according to a meta-analysis of 33 studies [26]. If DNA typing were performed before warfarin is prescribed, an estimated 85,000 serious bleeding events and 17,000 strokes could be avoided annually in the USA alone, saving over US$1 billion in healthcare spending [27]. The USA National Heart, Lung, and Blood Institute is currently sponsoring a prospective genotype-guided warfarin dosing protocol [28].

The goals of our study were to determine the frequencies of over- and under-anticoagulation can be serious, ranging from life-threatening bleeding to cerebrovascular emboli, and require intensive treatment. Major and fatal bleeding events occur at rates of 7.2 and 1.3 per 100 patient years, respectively, according to a meta-analysis of 33 studies [26]. If DNA typing were performed before warfarin is prescribed, an estimated 85,000 serious bleeding events and 17,000 strokes could be avoided annually in the USA alone, saving over US$1 billion in healthcare spending [27]. The USA National Heart, Lung, and Blood Institute is currently sponsoring a prospective genotype-guided warfarin dosing protocol [28].

The goals of our study were to determine the frequencies of SNPs in the CYP2C9 and VKORC1 genes in a cardiovascular population and the carrier prevalences for their combinatorial genotypes, and to gauge the impact of these polymorphisms on warfarin dosage using published algorithms.

Materials & methods
Patient cohort
As part of a study of dyslipidemias, outpatients at Hartford Hospital (Hartford, CT, USA) were recruited and provided written consent as approved by the Institutional Review Board (29). All patients were adults and unrelated. Ages ranged from 28 to 88 years, with an average age of 67 years. The ethnicities of the surveyed population were obtained from each participant’s self-report at recruitment. The ethnic composition of the survey obtained was 80% Caucasian, 10% African–American, 9% Hispanic and 1% Asian.

Laboratory analysis
A 5–10 ml blood sample treated with ethylenediaminetetraacetic (EDTA) was obtained at the time of routine lipid collection for each patient. DNA samples were extracted from whole blood using QIAamp DNA Blood Midi Kit (Qiagen, CA, USA) following the manufacturers protocol. Extracted DNA was stored at -80ºC in trishydroxymethylaminomethane (TRIS)-EDTA (TE) buffer. Quantification of DNA was performed by fluorescent staining of double-stranded DNA (PicoGreen® dsDNA Quantitation Kit, Molecular Probes, OR, USA). Fluorescent intensity was measured using a fluorescent micro-titer plate reader (POLARstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany).

DNA typing of the CYP2C9 and VKORC1 genes at 12 variable sites, five SNPs in CYP2C9 (Table 1) and seven SNPs in VKORC1 (Table 2), was performed at the Laboratory of Personalized Health (LPH), a division of Genomas Inc. (Hartford, CT, USA). The LPH is a high-complexity clinical DNA testing center licensed by the Connecticut Department of Health (CL-0644) and certified by the Centers for Medicare and Medicaid Services (ID# 07D1036625) under Clinical Laboratory Improvement Amendments (CLIA). The Tag-It™ Mutation Detection assays (Luminex Molecular Diagnostics, Toronto, Canada) were utilized for DNA typing [30]. These assays employed PCR to amplify selectively the desired genes without co-amplifying pseudogenes or other closely related sequences.

The kits use multiplexed allele-specific primer extension (ASPE) to identify small nucleotide variations including single base changes and deletions. In brief, a PCR-derived target DNA with two universally-tagged allele-specific primers whose 3’ ends define the alleles was used for each variation tested. A thermostable DNA polymerase was used for primer extension and biotin-deoxycytidine triphosphate (dCTP) label incorporation. Because the tagged allele-specific primers overlap the SNP site in the target DNA, only the correctly hybridized primers were extended to generate labeled products. Single-tagged ASPE primers were used to detect the presence of unique PCR fragments generated for the deletion and duplication gene rearrangements. Following ASPE, tagged, extended products labeled with biotin were captured by their tag complements (anti-tags), which had been chemically coupled to spectrally addressable polystyrene microspheres.
A fluorescent reporter molecule (streptavidin-phycoerythrin) was used to detect incorporated biotin. The fluorescent reporter signals generated for each bead population was measured on the Luminex xMAP™ system. (Luminex® Corp., TX, USA).

**Statistical analysis**

To explore the population-wide impact of the polymorphisms on warfarin dose, we classified patients by \(VKORC1\) and \(CYP2C9\) combinatorial genotypes. We calculated the expected reduction in dose for each combination, using the equations of Sconce et al. [13] and Zhu et al. [12].

First, warfarin dose was estimated for each combinatorial genotype, assuming a reference person of age 60 years and height 165 cm, weight 68.1 kg for a female, and height 177 cm, weight 78.3 kg for a male. The algorithms of Sconce et al. [13] and Zhu et al. [12] account for body size through inclusion of height and body weight, respectively. In our analysis, four values were generated for each combinatorial genotype: two values for the Sconce et al. [13] algorithm incorporating the reference male and female heights, and two values for the Zhu et al. [12] algorithm incorporating the respective weights.

The reduction in dose for each combinatorial genotype was calculated as the difference between the dose predicted for that combinatorial genotype and the dose predicted for the reference genotype \(CYP2C9\) *1/*1, \(VKORC1\) GG. The dose reductions for the reference man and woman were averaged separately within each combinatorial genotype for Zhu et al. [12], and for Sconce et al. [13]. The Zhu et al. [12] algorithm predicts a systematically greater warfarin dose reduction across combinatorial genotypes compared with Sconce et al. [13].

**Results**

Survey results for the \(CYP2C9\) SNPs are shown in Table 1. The frequency of the alleles *2 and *3 were 10.2 and 7.9%, respectively. With respect to warfarin metabolism, the *3 allele is deemed ‘highly deficient’, having a metabolic function of only 5% relative to the normal level. Table 2 presents the SNP frequencies for the \(VKORC1\) gene. The LPH tests for seven different \(VKORC1\) SNPs, but only the -1639A promoter allele was observed, at 34.7% frequency.

The carrier prevalence for our 127-patient survey, combining polymorphisms in both \(CYP2C9\) and \(VKORC1\) genes is shown in Figure 1. Patient

### Table 1. CYP2C9 alleles.

<table>
<thead>
<tr>
<th>Allele</th>
<th>DNA change</th>
<th>AA change</th>
<th>Frequency (%)</th>
<th>Allele count</th>
</tr>
</thead>
<tbody>
<tr>
<td>*1</td>
<td>Reference</td>
<td>Reference</td>
<td>81.9</td>
<td>208</td>
</tr>
<tr>
<td>*2</td>
<td>430C&gt;T</td>
<td>Arg 144 Cys</td>
<td>10.2</td>
<td>26</td>
</tr>
<tr>
<td>*3</td>
<td>1075A&gt;C</td>
<td>Ile 359 Leu</td>
<td>7.9</td>
<td>20</td>
</tr>
<tr>
<td>*4</td>
<td>1076T&gt;C</td>
<td>Ile 359 Tyr</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>*5</td>
<td>1080C&gt;G</td>
<td>Asp 360 Glu</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>*6</td>
<td>818delA</td>
<td>Frameshift</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Those observed among \(n = 254\) alleles from 127 individuals are shown in bold.

AA: Amino acid.

### Table 2. VKORC1 alleles.

<table>
<thead>
<tr>
<th>Allele</th>
<th>DNA change</th>
<th>AA change</th>
<th>Frequency (%)</th>
<th>Allele count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>Reference</td>
<td>Reference</td>
<td>62.6</td>
<td>159</td>
</tr>
<tr>
<td>-1639</td>
<td>G&gt;A</td>
<td>Promoter</td>
<td>37.4</td>
<td>95</td>
</tr>
<tr>
<td>85</td>
<td>G&gt;T</td>
<td>Val 29 Leu</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>121</td>
<td>G&gt;T</td>
<td>Ala 41 Ser</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>134</td>
<td>T&gt;C</td>
<td>Val 45 Ala</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>172</td>
<td>A&gt;G</td>
<td>Arg 58 Gly</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1331</td>
<td>G&gt;A</td>
<td>Val 66 Met</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3487</td>
<td>T&gt;G</td>
<td>Leu 128 Arg</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Those observed in \(n = 254\) alleles from 127 individuals of the present study are shown in bold.

AA: Amino acid.
counts for each category are presented in the caption. The patients without a polymorphism for either gene (noncarriers) accounted for 26.8% of the population. The percentage of patients with polymorphism in only one gene was 12.6% for CYP2C9 and 42.5% for VKORC1. Patients with one polymorphism in both genes accounted for 11.8% of the population. Patients who are carriers of three polymorphisms, single carriers in one gene and double carriers in the other, accounted for 4.7%. Carriers of four polymorphisms, double carriers in both genes, accounted for 1.6% of the population. 

Figure 2 depicts the projected effect of CYP2C9 and VKORC1 combinatorial genotypes on the warfarin dose reduction, based on two published equations [12,13] applied to the 127 patients in our survey. The width of each colored zone corresponds to the percentage of the cohort with a given combinatorial genotype. The top and bottom of the colored zones correspond to the ranges of predicted decreases in dose of warfarin associated with the combinatorial genotypes, according to the Zhu et al. [12] and Sconce et al. [13] formulae, respectively. Both yield step-wise dose reductions dependent on the number of polymorphisms.

In this study, 43.3% of patients were carriers of one polymorphism in CYP2C9 (*1*2 or *1*3) or VKORC1 (AG) and, relative to noncarriers, would require a dose decrease in the range of 1.0–1.6 mg/day. Carriers of two polymorphisms, one polymorphism in each gene or two in VKORC1, accounted for 23.6% of the population and would require a dose reduction in the range of 2.0 to 2.95 mg/day. Carriers of three polymorphisms (CYP2C9 *2*3, VKORC1 AG and CYP2C9 *1*2 or *1*3, VKORC1 AA) accounted for 4.7% of the population and would require a decrease in dose between 2.9 and 3.7 mg/day. Finally, carriers of four polymorphisms (two in each gene) accounted for 1.6% of the population and would require a decrease between 3.6 and 4.4 mg/day.
Discussion

The results from this study in Figure 1 demonstrate that 73.2% of the patients in our population were carriers of one or more CYP2C9 or VKORC1 polymorphisms resulting in deficient warfarin metabolism (CYP2C9) or sensitivity (VKORC1). Frequencies for each polymorphism were consistent with published literature values. Since only approximately a quarter of our study population is not a carrier of CYP2C9 or VKORC1 polymorphisms, the potential need for genotype-guided warfarin dosing in all cardiovascular patients can be discerned. Carriers of three or four polymorphisms accounted for 1.6% of the population. Remarkably, even with 127 patients in the study, two subjects were found to be carriers of four polymorphisms, with double polymorphisms in both CYP2C9 and VKORC1 genes.

The CYP2C9 and VKORC1 genes map to chromosomes 10q24 and 16p11.2, respectively. Hence, their polymorphisms would be expected to segregate independently in populations. Our results establish this independent assortment to be the case. The combined frequency of CYP2C9 *1*2 or *1*3 and VKORC1 GA for example, could be calculated to be [(18/127 + 14/127) X 59/127], or 0.117, which is in agreement with the observed carrier prevalence of 15/127, or 0.118, for the combinatorial genotype CYP2C9 *1*2 or *1*3, VKORC1 GA. While these combinatorial genotypes can be calculated, independent segregation should be confirmed, as each allele combination has a different effect on dosage adjustment. Our survey documents the existing high prevalence of combinatorial genotypes affecting warfarin dosing.

Figure 2. Predicted mean decreases in warfarin dose (mg/day) in the 127 patients from the surveyed population with reference to carrier status for polymorphisms in the CYP2C9 and VKORC1 genes.

The decreases were calculated as described in the methods using published formulae [12,13] that employ age, gender, weight, height, CYP2C9 genotype and VKORC1 genotype. The colored areas indicate ranges of predicted dose reductions. The top and bottom of the coloured zones represents the reductions according to the formulae of Zhu et al. [12] and Sconce et al. [13], respectively. The carrier prevalences and their respective combinatorial genotypes were: 26.8% noncarriers; 43.3% carriers of one polymorphism (33.1% with CYP2C9*1*1, VKORC1 GA and 10.2% with CYP2C9 either *1*2 or *1*3, VKORC1 GG); 23.6% carriers of two polymorphisms (11.8% with CYP2C9 either *1*2 or *1*3, VKORC1 GG; 9.4% with CYP2C9*1*1, VKORC1 AA; 2.4% with CYP2C9 either *2*2 or *2*3, VKORC1 GG); 4.7% carriers of three polymorphisms (3.1% with CYP2C9 either *1*2 or *1*3, VKORC1 AA; 1.6% with CYP2C9 either *2*2 or *2*3, VKORC1 GA); and 1.6% carriers of four polymorphisms (CYP2C9*2*2, VKORC1 AA).
As shown quantitatively in Figure 2, when applied to dosing algorithms, the combinatorial genotypes shed valuable information on the frequency and magnitude of potential genotype-guided adjustments in a typical population of cardiovascular patients. To estimate the effect of these genotypes on clinical dosing practices, we calculated the warfarin dosage for each patient based on CYP2C9 *2/*3 and VKORC1 -1639G>A alleles, age, gender and physical attributes using the published algorithms of Zhu et al. [12] and Sconce et al. [13]. Clearly, the patients who stand to benefit most from genotyping are those found to have the greatest number of deficient polymorphisms. Enrichment for risk-associated combinatorial genotypes may be examined in patients with reported adverse events. Recent studies demonstrate that carriers of a combination of CYP2C9 and VKORC1 polymorphisms have a significantly increased risk of severe over-anticoagulation when compared with patients who carry polymorphisms in only a single gene [31,32].

For the present cohort, the potential impact of the genotype test results on the population burden of anticoagulation therapy risk can be seen in Figure 2. As the beginning of a practical mnemonic, it can be appreciated that each polymorphism in CYP2C9 and VKORC1 for which the patient is a carrier entails an approximately proportional dose reduction. We found the dose reductions to be in excellent agreement with those derived for patients aged 60 years from a recently published nomogram, which predicted effective warfarin dose ranges for combinatorial genotypes for the VKORC1 6853 G>C SNP and CYP2C9 *2/*3 in patients aged 40–85 years [33]. The VKORC1 SNPs at -1639 and 6853 are in linkage disequilibrium, serving as equivalent markers for the VKORC1 haplotypes A and B [24]. In the present study and that of Caldwell et al. [33], the reductions in warfarin dose for carriers of one, two and three polymorphisms compared with noncarriers were in the ranges 15–29%, 34–47% and 55–62%, respectively.

Discrepancies in warfarin management from the genotype-guided doses may increase the risk of overdosing and bleeding complications [34,35], especially during the initiation of warfarin therapy [36,37]. Studies in Caucasian populations show that the CYP2C9 polymorphisms are associated with a two- to three-fold increased risk of bleeding during warfarin induction [9,10,38,39], but not during long-term therapy [40].

This study has some limitations. The universe of alleles for CYP2C9 and VKORC1 genes continues to expand with genomic sequencing efforts in various populations. Nevertheless, we believe our multiplexed panel of five and seven alleles for CYP2C9 and VKORC1, respectively, addresses common and clinically relevant alleles for these genes. Another limitation is the lack of dosing algorithms applicable to ethnographically diverse urban populations. We are currently pursuing targeted recruitment and dosing algorithm development for the Hispanic population. A recent study in African–Americans has demonstrated increased risk of hemorrhage during long-term therapy in patients with CYP2C9 minor variants [36]. In Asian populations, where VKORC1 polymorphisms are more frequent than in Caucasians [41,42], genotype-guided warfarin dosing may also be clinically valuable [37].

DNA typing for CYP2C9 and VKORC1 polymorphisms has the potential to enhance the standard of care in warfarin management. Websites and other portals now facilitate the application of a warfarin dosing algorithm by physicians, taking into account the CYP2C9 and VKORC1 genotypes as well as age, gender, smoking history and concomitant medications [43]. Warfarin dosing represents a current working model for the practice of personalized healthcare increasingly dependent on DNA typing and managing therapy according to combinatorial genotype. DNA-guided pharmacotherapy holds great potential to enhance patient safety based on each individual’s innate drug metabolism and sensitivity [101].

Acknowledgements
The authors roles in this work are listed in order to clarify possible conflicts of interest: GR conceived of the study, and participated in its design and in drafting the manuscript; PDT and AHBW participated in the design and conduct of the study; MK and GY performed DNA analysis; AW performed statistical analyses; DV, RLS, JD, and BB prepared the manuscript; and TRH and CMW participated in data interpretation. We thank Darrin D’Agostino, D.O., for his contributions to the manuscript.

Financial & competing interests disclosure
This research was funded by grants from the Hartford Hospital Research Administration and by Genomas internal research and development funds. All work, including the conception and design of the study, the collection of the biological samples from patients, DNA typing and writing of the manuscript, was completed at Hartford Hospital and Genomas,
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Inc. The following relevant conflicts of interest exist: GR is founder and President of Genomas, Inc., AW and MK are employees and GY is a former employee of Genomas Inc. RLS and TRH are consultants for Genomas, Inc. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Executive summary

- In August 2007, the US FDA revised the warfarin label to include pharmacogenomic information on prescription and clinical consequences of CYP2C9 and VKORC1 gene polymorphisms.

- There is a need to translate this guidance into public health impact based on the prevalence of combinatorial genotypes predicting dose adjustments in substantial segments of the population with cardiovascular morbidity.

- 127 outpatients with dyslipidemia, a diagnosis that places them at risk for cardiovascular disease and raises the potential for warfarin therapy, were genotyped for five DNA sequence variants in the CYP2C9 gene and for seven in the VKORC1 gene.

- The individual gene allele frequencies were 10.2, 7.9 and 37.4% for the functionally deficient G>A warfarin metabolism catalysed by the VKORC1 gene.

- The dosing profile constructed for this dyslipidemic population illustrated the large segments of the population potentially requiring warfarin therapy, were genotyped for five DNA sequence variants in the CYP2C9 gene and for seven in the VKORC1 gene. The individual gene allele frequencies were 10.2, 7.9 and 37.4% for the functionally deficient G>A warfarin metabolism catalysed by the VKORC1 gene.

- The combination of genotyping and pharmacogenomics led to a dosing profile constructed for this dyslipidemic population illustrating the large segments of the population potentially requiring warfarin therapy and of the potential benefits of genotype-guided warfarin dosing.

- In August 2007, the US FDA revised the warfarin label to include pharmacogenetic information on prescription and clinical consequences of CYP2C9 and VKORC1 gene polymorphisms.

Bibliography

Papers of special note have been highlighted as such. Readers are encouraged to consult the references cited in the text and to read the original articles for a comprehensive understanding of the subject matter.

PERSONALIZED MEDICINE IN ACTION – Ruano, Thompson, Villagra et al.


Website

101. Warfarin Dosing. Barnes-Jewish Hospital, Washington University Medical Center www.warfarindosing.org/Source/Home.aspx